

Mast Cell-Mediated Antigen Presentation Regulates CD8⁺ T Cell Effector Functions

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SUMMARY

The characteristics, importance, and molecular requirements for interactions between mast cells (MCs) and CD8⁺ T cells have not been elucidated. Here, we demonstrated that MCs induced antigen-specific CD8⁺ T cell activation and proliferation. This process required direct cell contact and MHC class I-dependent antigen cross-presentation by MCs and induced the secretion of interleukin-2, interferon- γ , and macrophage inflammatory protein-1 α by CD8⁺ T cells. MCs regulated antigen-specific CD8⁺ T cell cytotoxicity by increasing granzyme B expression and by promoting CD8⁺ T cell degranulation. Because MCs also upregulated their expression of costimulatory molecules (4-1BB) and released osteopontin upon direct T cell contact, MC-T cell interactions probably are bidirectional. In vivo, adoptive transfer of antigen-pulsed MCs induced MHC class I-dependent, antigen-specific CD8⁺ T cell proliferation, and MCs regulated CD8⁺ T cell-specific priming in experimental autoimmune encephalomyelitis. Thus, MCs are important players in antigen-specific regulation of CD8⁺ T cells.

INTRODUCTION

Mast cells (MCs) are well recognized as key effector cells in immunoglobulin E (IgE)-associated immune responses and as prototypic regulators of innate immunity (Marshall, 2004; Metz and Maurer, 2007; Orinska et al., 2007; Galli et al., 2008). However, evidence that MCs also play an important regulatory role in adaptive immunity is steadily accumulating (Galli et al., 2005; Stelekati et al., 2007; Sayed et al., 2008). For example, MCs modulate the phenotype and function of adaptive immunity protagonists, such as B cells, dendritic cells (DCs), and T cells via direct cell contact, release of exosomes, or secretion of soluble factors (Caron et al., 2001; Gauchat et al., 1993; Kashiwakura et al., 2004; Nakae et al., 2005; Skokos et al., 2003; Tkaczyk et al., 2000).

MCs actively regulate T cell migration. MCs can migrate from the site of allergen encounter to the draining lymph nodes, in which they recruit T cells (Wang et al., 1998). MCs also recruit T cells at peripheral sites of inflammation by secreting chemotactic molecules, such as MIP-1 β , RANTES, and interleukin-16 (IL-16) (Burd et al., 1989; Okumura et al., 2003; Rumsaeng et al., 1997). MC-derived tumor necrosis factor- α (TNF- α) induces chemotaxis of circulating T cells into the draining lymph nodes during bacterial infection (McLachlan et al., 2003). Fc ϵ RI-mediated degranulation of MCs results in chemotaxis of effector CD8⁺ T cells via leukotriene B₄ (LTB₄) secretion (Ott et al., 2003). CD8⁺ T cell recruitment is also induced upon Toll-like receptor 3 (TLR3)-dependent activation of MCs in vivo (Orinska et al., 2005). Collectively, this evidence suggests that MCs regulate a wide, as yet to be elucidated range of MC-CD8⁺ T cell functions.

The concept that MCs may induce T cell responses initially arose after the observation that cultured MC progenitors and primary MCs (Wong et al., 1982; Banovac et al., 1989) can express surface and intracellular major histocompatibility complex (MHC) class II molecules. Subsequently, MHC class II and expression of costimulatory molecules was detected on mouse, rat, and human MCs. MCs were shown to induce proliferation of CD4⁺ T cell lines upon presentation of MHC class II-restricted antigens in vitro (Fox et al., 1994; Frandji et al., 1993; Poncet et al., 1999) as well as proliferation of a CD8⁺ T cell hybridoma line upon phagocytosis of living bacteria (Malaviya et al., 1996). Costimulatory molecules of the B7 family and the TNF-TNFR families (e.g., 4-1BB and 4-1BBL) were identified on mouse bone marrow-derived MCs (BMMCs) and human primary MCs (Kashiwakura et al., 2004; Nakae et al., 2006). In vivo, MCs modulate T cell responses in murine experimental autoimmune encephalomyelitis (EAE), the standard animal model for multiple sclerosis, in which both CD8⁺ T cells and MCs are recognized key players (Secor et al., 2000; Tanzola et al., 2003; Gregory et al., 2005; Sayed et al., 2008).

These findings suggested, but did not prove, that MCs can interact with T cells in an antigen-specific manner. Here, we show that MCs induce CD8⁺ T cell activation and proliferation, cytokine secretion, and cytotoxicity in an antigen-dependent manner and that, vice versa, this interaction also modulates MC activities.

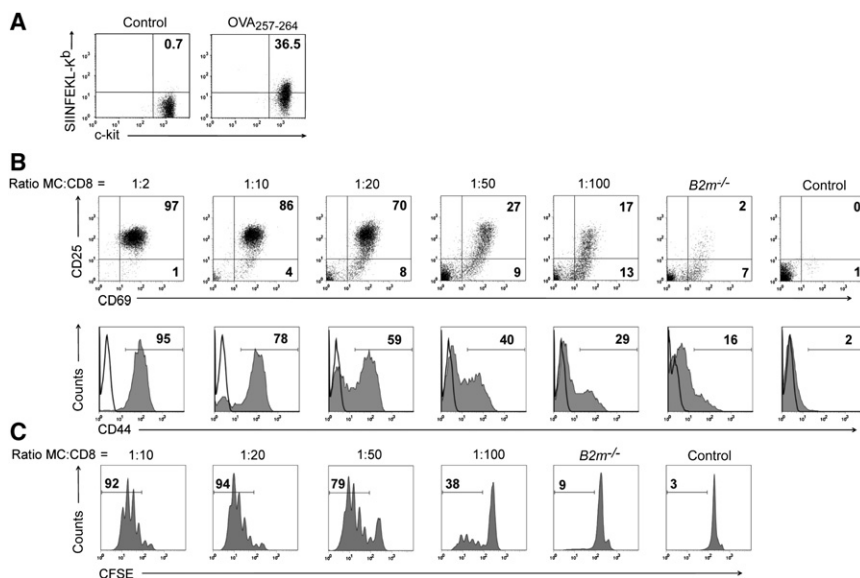


Figure 1. MCs Induce Antigen-specific CD8⁺ T Cell Activation and Proliferation In Vitro

(A) BMMCs were pulsed with OVA₂₅₇₋₂₆₄ peptide (right panel) or left untreated (left panel) for 2 hr and stained with an antibody recognizing OVA₂₅₇₋₂₆₄ (SINFEKL) in complex with MHC class I molecules (H-2K^b). One representative experiment from three is shown.

(B and C) BMMCs were pulsed with OVA₂₅₇₋₂₆₄ peptide and cultured with OT-I transgenic CD8⁺ T cells for 48 hr. As negative control, CD8⁺ T cells were cultured with B2m^{-/-}, OVA₂₅₇₋₂₆₄-pulsed BMMCs or unpulsed BMMCs (control). As shown in (B), CD8⁺ T cell activation was determined by analysis of surface expression of CD69 and CD25 (upper panel) and CD44 (lower panel) by flow cytometry. Staining with control isotype-matched antibodies is shown in bold black lines. Representative results of six independent experiments are shown. As shown in (C), proliferation of CD8⁺ T cells was detected by CFSE dilution. Representative results of three to five independent experiments are shown.

RESULTS

MCs Present Antigenic Peptides via MHC Class I

We first tested whether MCs are able to take up exogenous peptides, to generate complexes with MHC class I molecules, and to express them on the cell surface (Porgador et al., 1997). Specific complexes of OT-I peptides and MHC class I molecules (H-2K^b) were indeed recognized by 25-D1.16 antibodies on the surface of BMMCs after preincubation with the model antigenic peptide (OVA₂₅₇₋₂₆₄, SINFEKL) (Figure 1A). This was not seen on BMMCs without peptide preincubation.

In order to probe whether MCs induce CD8⁺ T cell responses in an antigen-dependent manner, we cocultured OVA₂₅₇₋₂₆₄-loaded BMMCs with T cell receptor- (TCR-) transgenic (OT-I), purified CD8⁺ T cells at various MC:CD8⁺ T cell ratios. CD8⁺ T cell activation was analyzed by flow cytometry. After 48 hr, antigen-pulsed MCs activated CD8⁺ T cells (Figure 1B), as shown by a marked upregulation of the T cell activation markers CD69, CD25, and CD44 on CD8⁺ T cells. All three T cell activation markers showed the same dynamics with maximal upregulation noted at a 1:2 ratio of MC:CD8⁺ T cells. However, even at a much lower ratio of MC:CD8⁺ T cells (1:10), up to 90% ± 4% of CD8⁺ T cells displayed an activated phenotype (n = 6). The lowest tested ratio of MC:CD8⁺ T cells = 1:100 still sufficed to induce antigen-specific activation of 29% ± 9% of the CD8⁺ T cells (n = 6) (Figure 1B). Thus, MCs are highly effective activators of CD8⁺ T cells in vitro. For subsequent experiments, the MC:CD8⁺ T cell ratio of 1:10 was selected, unless indicated otherwise. When MCs were cultured with CD8⁺ T cells in the absence of OVA₂₅₇₋₂₆₄, no CD8⁺ T cell activation was seen (Figure 1B, control), demonstrating that the MC-induced CD8⁺ T cell activation was indeed antigen dependent. In addition, OVA₂₅₇₋₂₆₄-pulsed BMMCs failed to activate primary purified CD8⁺ T cells from wild-type (WT) mice (data not shown), indicating that activation of CD8⁺ T cells by MCs was antigen specific.

To explore whether the observed MC-T cell interaction depends on the expression of MHC class I on MCs, we used

BMMCs derived from beta-2-microglobulin (β₂m)-deficient (B2m^{-/-}) mice, which do not express functional MHC class I (Zijlstra et al., 1990), for coculture with CD8⁺ T cells. Compared to MHC class I-competent WT MCs, the B2m^{-/-} MCs induced only a minimal activation of CD8⁺ T cells (4.6% ± 2.1%, n = 4), with CD69, CD25, and CD44 surface expression as activation markers (Figure 1B). This indicates that the bulk of CD8⁺ T cell activation induced by MCs was due to antigen presentation via functional MHC class I molecules.

Next, we examined the proliferation of CD8⁺ T cells by incubating OVA₂₅₇₋₂₆₄-pulsed MCs with CFSE-labeled OT-I T cells. Maximal proliferation was detected at a ratio of MC:CD8⁺ T cells = 1:20, with 92% ± 2% (n = 3) (Figure 1C). At the lowest tested ratio (1:100), a total of 57% ± 17% (n = 4) of the CD8⁺ T cells still was in the proliferation pool, whereas no T cell proliferation was detected in the presence of unpulsed MCs or with B2m^{-/-}, OVA₂₅₇₋₂₆₄-pulsed MCs (Figure 1C). Thus, MCs induced antigen-specific proliferation of CD8⁺ T cells in an MHC class I-dependent manner.

MC-CD8⁺ T Cell Coculture Stimulates Cytokine and MIP-1α Release by CD8⁺ T Cells

Because the secretion of defined cytokines is a key effector function of activated CD8⁺ T cells, we investigated the cytokine milieu generated upon antigen-dependent MC-CD8⁺ T cell contact in vitro. High amounts of IL-2, IFN-γ, and MIP-1α (Figure 2A), as well as lower amounts of TNF-α (0.31 ± 0.18 ng/ml) (n = 3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (0.4 ± 0.12 ng/ml) (n = 3) (data not shown), were measured in supernatants after antigen-specific MC-CD8⁺ T cell contact. In contrast, hardly any or no production of IL-4, IL-5, IL-10, and IL-12 was seen (data not shown).

IL-2, IFN-γ, TNF-α, and MIP-1α were detected intracellularly in CD8⁺ T cells upon MC-mediated antigen-dependent activation (Figure S1 available online), at a percentage of 5.5% ± 2.9% (n = 10), 3.1% ± 1.5% (n = 10), 7.5% ± 0.57% (n = 4), and 19.3 ± 8.7% (n = 3) respectively. Furthermore, when

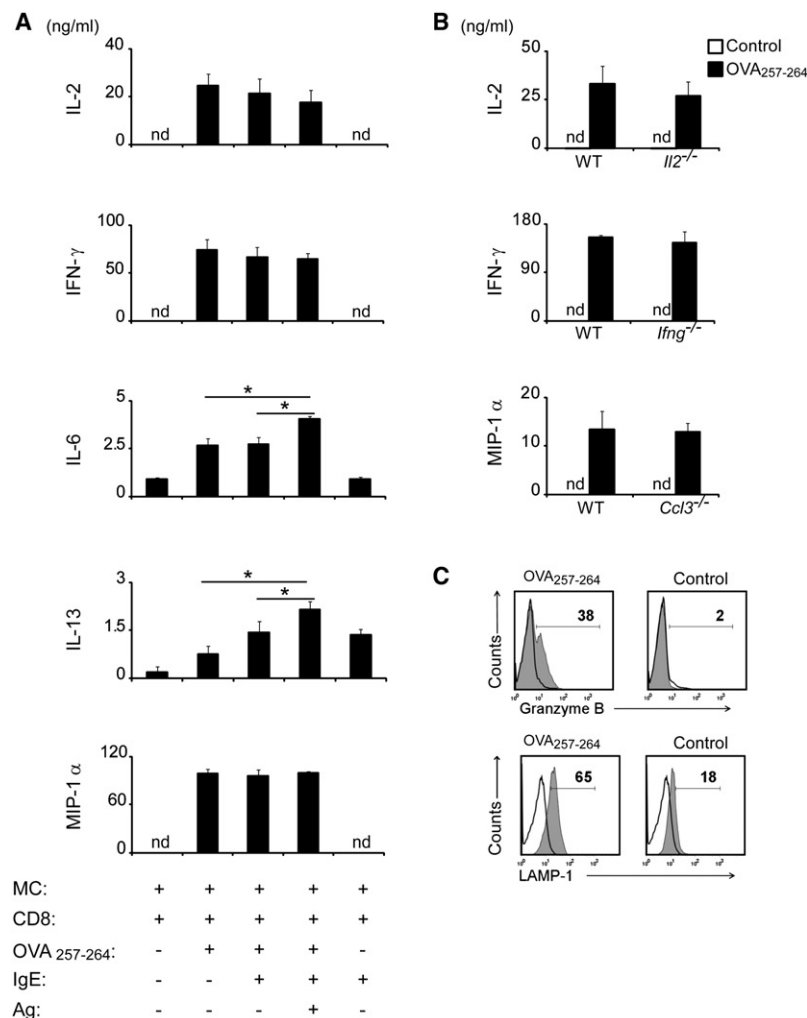


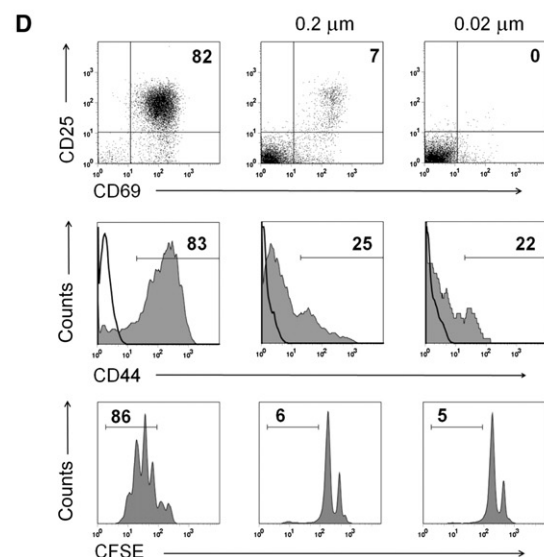
Figure 2. MCs Stimulate Antigen-Specific CD8⁺ T Cell Responses

BMMCs were pulsed with OVA₂₅₇₋₂₆₄ peptide in the presence or absence of IgE and antigen (Ag) and cultured with OT-I transgenic CD8⁺ T cells for 48 hr. CD8⁺ T cells were incubated with antigen-pulsed MCs from either WT or deficient mice (*Il2*^{-/-}, *Ifng*^{-/-}, *Ccl3*^{-/-}) in a 1:10 ratio. As negative control, CD8⁺ T cells were cultured with unpulsed BMMCs.

(A and B) Cytokine concentrations in the coculture supernatants were determined by ELISA. Mean values $\pm$ SD of three independent experiments are shown. nd, non detectable.

(C) The cytotoxic potential of CD8⁺ T cells was measured by intracellular flow cytometry staining for granzyme B (gated on CD8⁺ T cells) (left panel) and cell-surface expression of LAMP-1 (gated on viable CD3⁺ T cells) (right panel). Representative results of three independent experiments are shown. * $p \leq 0.0005$.

(D) The antigen-specific CD8⁺ T cell responses induced by MCs are cell-contact dependent. BMMCs were pulsed with OVA₂₅₇₋₂₆₄ peptide and cultured with CD8⁺ T cells for 48 hr. Direct cell contact between MCs and CD8⁺ T cells was inhibited by the presence of 0.2 μ m or 0.02 μ m-anopore membranes, as indicated. Activation of CD8⁺ T cells was measured by CD69, CD25 (upper panel), and CD44 (middle panel) expression by flow cytometry. Staining with control isotype matched antibodies is shown in bold black lines. Proliferation of CD8⁺ T cells was visualized by CFSE dilution (lower panel). Representative results of three independent experiments are shown.



OVA₂₅₇₋₂₆₄-pulsed BMMCs derived from IL-2-, IFN γ -, or MIP-1 α -deficient mice were cocultured with OT-I T cells, no significant differences in IL-2, IFN γ , and MIP-1 α concentrations were found between the cocultures that used wild-type (WT) compared to cytokine-deficient BMMCs (Figure 2B). Thus, activated CD8⁺ T cells were the primary source of these cytokines.

Fc ϵ RI Stimulation Does Not Affect MC-Mediated CD8⁺ T Cell Activation

Next, we investigated whether an Fc ϵ RI-mediated MC activation would both affect the MC-induced, antigen-specific CD8⁺ T cell activation and switch the CD8⁺ T cell response toward a Th2 cell type. To address this question, we activated BMMCs via Fc ϵ RI engagement (IgE+Ag), pulsed them with antigen (OVA₂₅₇₋₂₆₄), and subsequently cocultured them with OT-I T cells.

Fc ϵ RI-mediated BMMC degranulation by itself did not affect CD8⁺ T cell activation (Figure S2). However, this was associated with augmented concentrations of IL-6 and IL-13 in the supernatants, whereas those of IL-2 and IFN γ remained unchanged (Figure 2A). Therefore, despite stimulation of a classical MC activation pathway associated with the production of Th2 cell-type cytokines, the CD8⁺ T cell response remained skewed toward a Th1 cell-type secretory response pattern.

TLR Ligands Enhance MC-Induced CD8⁺ T Cell Activation

Mature MCs are most prominently found as resident cells in peripheral tissue locations where the organism's chance of encountering invading pathogens is highest. Indeed, stimulation of MCs via TLR3 (e.g., by poly IC) mediates CD8⁺ T cell recruitment in vivo (Orinska et al., 2005). Therefore, we hypothesized that the exposure of MCs to TLR ligands might modulate the capacity of MCs to activate CD8⁺ T cells. We found that TLR-activation of MCs by LPS or poly I:C enhanced the capacity of MCs to activate CD8⁺ T cells and to increase both MC surface expression of MHC class I molecules and their IL-2 cytokine secretion (Figure S3). Thus, invading pathogens may enhance the MHC class-I dependent antigen presentation of resident MCs.

Contact with Antigen-Specific MCs Enhances CD8⁺ T Cell Cytotoxic Potential and Degranulation

The amount of granzyme B (Gzmb) in CD8⁺ T cells upon interaction with antigen-presenting MCs was analyzed by flow cytometry as an indicator of CD8⁺ T cell-mediated cytotoxicity. Intracellular Gzmb expression was increased by 27% $\pm$ 9% (n = 9) in CD8⁺ T cells after antigen-mediated activation by MCs, a prerequisite for subsequent efficient cytolytic T cell activities (Figure 2C).

Next, we examined whether CD8⁺ T cell exocytosis was enhanced by measuring surface expression of lysosomal-associated membrane protein-1 (LAMP-1) on CD8⁺ T cells after activation by MCs (Burkett et al., 2005). Indeed, CD8⁺ T cells showed markedly increased surface LAMP-1 expression after activation with antigen-pulsed MCs (Figure 2C). Thus, at least in vitro, BMMCs increased the cytotoxic potential of CD8⁺ T cells by increasing their intracellular content of Gzmb and by inducing their degranulation in an antigen-specific manner.

Direct Cell-Cell Contact Is Essential for Antigen-Dependent MC-CD8⁺ T Cell Interaction

To further dissect the mechanistic requirements for antigen-specific MC-CD8⁺ T cell interactions, we investigated whether direct cell-contact is essential for CD8⁺ T cell activation by MCs. OVA₂₅₇₋₂₆₄-pulsed MCs were cultured in free cell contact with CD8⁺ T cells or in the presence of two kinds of semipermeable membranes: one that allows the passive diffusion of any soluble factor (0.2 μ m anopore membrane) or one (0.02 μ m membrane) that excludes the diffusion of exosomes. Both types of membranes completely abrogated the subsequent activation (upper and middle row) and proliferation (lowest row) of CD8⁺ T cells. This documented the necessity of direct cell-cell contact between MCs and CD8⁺ T cells for activation and proliferation of CD8⁺ T cells (Figure 2D). Although our data do not question the principle that MCs can regulate the phenotype and function of adaptive immune cells by releasing exosomes loaded with specific antigens (Skokos et al., 2003), our current results suggest that soluble factors (including exosomes) alone are insufficient to induce antigen-specific activation of CD8⁺ T cells by MCs.

MCs Induce Antigen-Specific CD8⁺ T Cell Responses upon *Listeria monocytogenes* Infection

MCs play a dominant role in various host defense mechanisms (Metz and Maurer, 2007; Galli et al., 2008). To explore whether MCs can stimulate CD8⁺ T cell responses upon infection with intracellular bacteria, we infected BMMCs with *Listeria monocytogenes*. BMMCs could be infected by *L. monocytogenes* at multiplicity of infection rates of 1:1 to 100:1 (Figure S4). To investigate potential CD8⁺ T cell responses induced by MCs upon their infection by *L. monocytogenes*, we used recombinant *L. monocytogenes*-OVA (Foulds et al., 2002).

BMMCs were infected with *L. monocytogenes*-OVA, extracellular bacteria were killed with gentamycin, and the MCs were cocultured with purified OT-I T cells. BMMCs that had been infected with *L. monocytogenes*-OVA activated CD8⁺ T cells, as shown by increased CD69 and CD25 surface expression (21.6% $\pm$ 12.7%, n = 5) (Figure 3A). This was in striking contrast to *L. monocytogenes*-infected BMMCs and to untreated BMMCs (control), which failed to activate the transgenic CD8⁺ T cells. Similarly, OVA-specific proliferation of the CD8⁺ T cells was observed upon coculture with *L. monocytogenes*-OVA-infected BMMCs (35.0% $\pm$ 15.4%, n = 5), but not upon coculture with *L. monocytogenes*-infected or untreated BMMCs. Finally, no activation or proliferation was observed when CD8⁺ T cells were cocultured with *L. monocytogenes*-OVA-infected, B2m^{-/-} BMMCs (Figure 3A). Thus, the antigen-specific activation and proliferation of CD8⁺ T cells induced by *L. monocytogenes*-OVA-infected MCs was MHC class I dependent. These results demonstrate that MCs can internalize, process, and present bacterial antigens and thus induce antigen-specific activation and proliferation of CD8⁺ T cells.

Peritoneal MCs Induce Antigen-Specific Activation of CD8⁺ T Cells upon Uptake of OVA In Vitro

In order to verify that primary MCs isolated from the peritoneal cavity internalize, process, and present antigens and therefore are capable of modulating CD8⁺ T cell activities, we incubated peritoneal MCs with OVA protein for 14 hr and cultured them

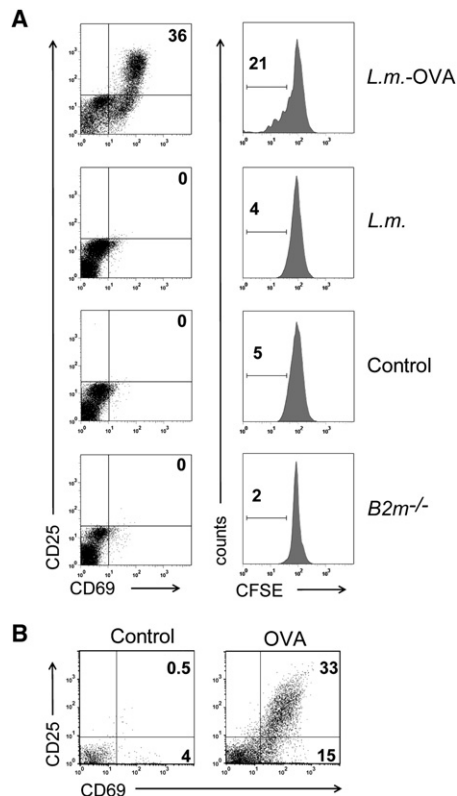


Figure 3. MCs Induce Antigen-Specific CD8⁺ T Cell Responses upon Processing of OVA

(A) BMMCs were infected with *L. monocytogenes*-OVA (*L.m.-OVA*) or *L. monocytogenes* (*L.m.*) or left untreated (control). As additional negative control, *B2m^{-/-}* BMMCs were infected with *L. monocytogenes*-OVA (*B2m^{-/-}*). BMMCs were cocultured with OT-I CD8⁺ T cells at a ratio of MC:CD8 = 1:2. Activation of CD8⁺ T cells was measured by surface expression FACS analysis of CD69 and CD25. Proliferation of CD8⁺ T cells was measured by CFSE dilution. Representative results of two to four independent experiments with at least four different BMMC cultures are shown.

(B) Peritoneal MCs (PMCs) were sorted by flow cytometry, incubated with OVA protein, and cocultured with CD8⁺ T cells at a ratio of MC:CD8 = 1:10. Activation of CD8⁺ T cells was measured by flow cytometric analysis of surface expression of CD69 and CD25. Representative results of three independent experiments are shown.

with OT-I transgenic CD8⁺ T cells. Subsequently, CD8⁺ T cell activation was analyzed by flow cytometry. Peritoneal MCs activated 35.3% ± 18.6% (n = 5) of the CD8⁺ T cells, whereas unpulsed peritoneal MCs failed to do so (Figure 3B). Peritoneal macrophages, used as a positive control, induced a similar activation of CD8⁺ T cells (26.3% ± 7.3% [n = 6]) compared to the T cell response obtained with peritoneal MCs (data not shown). Thus, primary MCs may cross-present extracellular antigens and activate CD8⁺ T cells as efficiently as peritoneal macrophages.

MCs Induce Antigen-Specific Proliferation of Primary CD8⁺ T Cells In Vivo

Next, the ability of MCs to induce antigen-specific CD8⁺ T cell responses in vivo was examined by adoptive transfer experiments. OVA₂₅₇₋₂₆₄-pulsed BMMCs were administered intraperitoneally to WT mice, then CFSE-labeled, OT-I transgenic CD8⁺ T cells were injected. Only OVA₂₅₇₋₂₆₄-pulsed BMMCs induced

the proliferation of CD8⁺ T cells in vivo (p = 0.002) (Figure 4A). OVA₂₅₇₋₂₆₄-pulsed BMMCs were used as a positive control (Figure S5). Because a comparable stimulation of CD8⁺ T cell proliferation by the transfer of OVA₂₅₇₋₂₆₄-pulsed MCs was observed in WT (30.3% ± 14.9%, n = 6) and in *B2m^{-/-}* mice (36.5% ± 13.3%, n = 6), this essentially excluded the possibility that other resident cells that present MHC class I-dependent antigens markedly influenced the CD8⁺ T cell proliferative response. This also underscored the key role of the transferred MCs for this T cell stimulation.

MCs Induce Antigen-Specific Proliferation of CD8⁺ T Cell to MOG In Vitro and In Vivo

Next, we tested the efficacy of MCs in regulating a polyclonal CD8⁺ T cell response against myelin oligodendrocyte glycoprotein (MOG), a key T cell antigen in EAE. MCs were pulsed with the MOG peptide (MOG₃₅₋₅₅) and incubated with MOG-primed CD8⁺ T cells in the presence of IL-2. The proliferative response of MOG-primed CD8⁺ T cells was tested. MOG₃₅₋₅₅-pulsed MCs induced a substantial, antigen-specific CD8⁺ T cell proliferation, as indicated by CFSE dilution of MOG-primed CD8⁺ T cells (Figure 4B).

To probe the in vivo relevance of these newly identified MC-T cell interactions, we employed the EAE model to test whether MCs affect CD8⁺ T cell effector functions in a MOG-specific manner. *W^{sh}/W^{sh}* mice were purposely chosen as MC-deficient mice strain because they lack MCs but have normal numbers and ratios of CD4⁺, CD8⁺, and CD11c⁺ cells in the spleen (Figure S6). WT and *W^{sh}/W^{sh}* mice were immunized with the MOG₃₅₋₅₅ peptide, which contains multiple CD4⁺ and CD8⁺ T cell epitopes (Sun et al., 2001), and spleens were harvested at day 7 after immunization. Splenocytes were labeled with CFSE and restimulated ex vivo with MOG₃₅₋₅₅.

As shown in Figure 4C, only the CD8⁺ MOG₃₅₋₅₅ T cell subset from *W^{sh}/W^{sh}* mice displayed a significantly reduced proliferation, whereas MOG₃₅₋₅₅-specific CD4⁺ T cells proliferated comparable to the ones obtained from immunized WT mice. Furthermore, the addition of BMMCs derived from WT mice to the splenocyte cultures increased the total rate of proliferation in both WT and *W^{sh}/W^{sh}* spleen cultures (Figure S6), indicating that the impaired CD8⁺ T cell proliferation was not caused by an intrinsic CD8⁺ T cell defect. Taken together, our results provide direct evidence that MCs contribute to CD8⁺ T cell-specific priming in EAE in vivo.

MC Reconstitution in *W^{sh}/W^{sh}* Mice Restores EAE Clinical Disease Severity and CD8⁺ T Cell Response

To directly evaluate the in vivo role of MCs in mediating an antigen-specific CD8⁺ T cell immune response against MOG, we elicited EAE in WT, *W^{sh}/W^{sh}*, and *W^{sh}/W^{sh}* mice reconstituted with WT BMMCs, assessed the clinical EAE features in all mice (onset of the clinical symptoms, disease incidence, and clinical score), and studied EAE immunopathology. Reconstitution of MCs in the spleen was documented by histochemistry (Figure S7B).

As shown in Figure 5A and Table 1, *W^{sh}/W^{sh}* mice developed significantly less severe disease than WT mice, as indicated by lower daily mean clinical scores (p < 0.05) and mean high score (p < 0.03). However, disease onset, incidence, and lethality were similar to that of WT controls. These data show that MCs control disease severity, but are not indispensable for disease

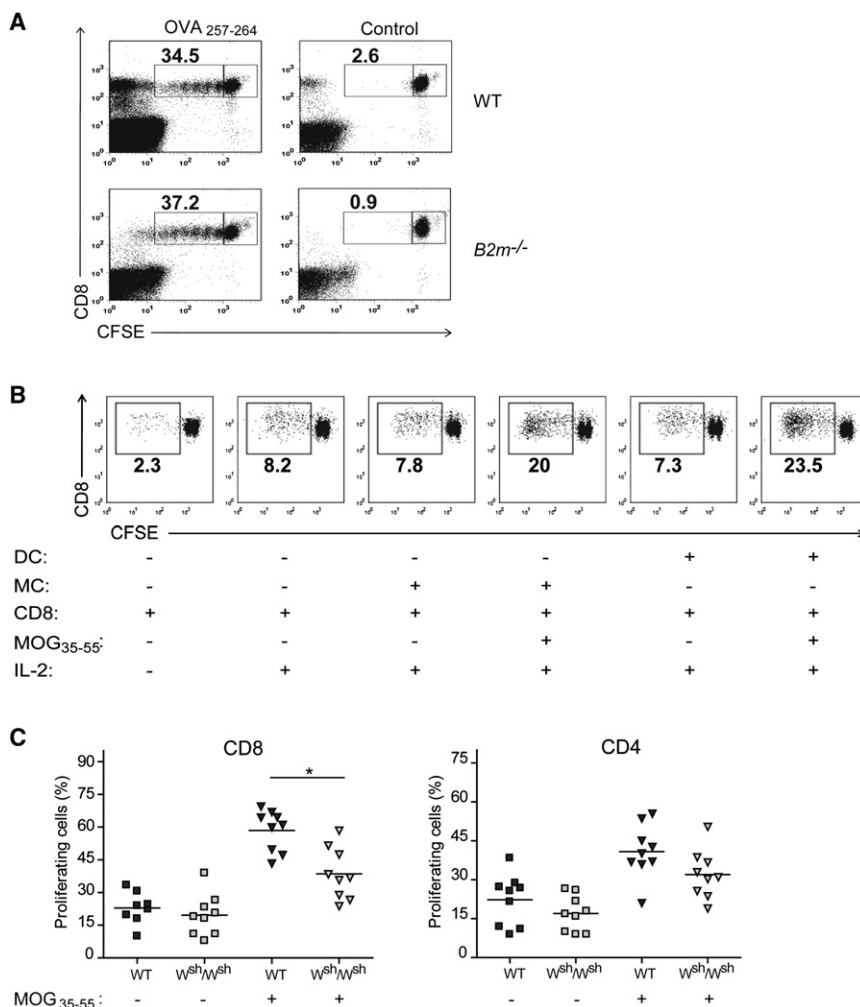


Figure 4. MCs Induce Antigen-Specific CD8⁺ T Cell Responses In Vitro and In Vivo

(A) A total of 5×10^6 BMMCs were pulsed with OVA₂₅₇₋₂₆₄ peptide or left untreated (control) and injected intraperitoneally into WT C57BL/6 or *B2m*^{-/-} recipients, as indicated. Twenty-four hours later, 8×10^6 purified CFSE-labeled, OT-I CD8⁺ T cells were transferred intraperitoneally. Four days later, peritoneal cells were isolated and analyzed for proliferation of the transferred CD8⁺ T cells by CFSE (gated on viable lymphocytes). Representative results of three independent experiments with at least seven mice per group are shown.

(B) BMMCs and BMDs (used for comparison) were loaded with MOG₃₅₋₅₅ peptide and incubated with CD8⁺ T cells isolated from MOG immunized mice. IL-2 was added after 3 days of coculture. Proliferation of CD8⁺ T cells was measured by analysis of CFSE dilution. Representative results of three independent experiments are shown.

(C) Spleens from MOG₃₅₋₅₅-immunized MC-deficient animals and controls were isolated and analyzed. Splenocytes were incubated with MOG₃₅₋₅₅ peptide. CD8⁺ and CD4⁺ T cell proliferation was measured by analysis of CFSE dilution. ($n = 8-9$). Results are representative of two to three independent experiments. * $p = 0.0011$.

development. Reconstitution of MCs in *W^{sh}/W^{sh}* mice by adoptive transfer of WT BMMCs restored the EAE clinical score (Figure 5A and Table 1).

Histochemistry of CNS (Figure 5B) and spinal cord (data not shown) 27 days after immunization revealed a comparable focal inflammatory cell infiltration between WT and WT BMMCs-reconstituted *W^{sh}/W^{sh}* mice. Twenty-seven days after immunization, brains and spinal cords were recovered and infiltrating cell subsets were analyzed by flow cytometry. The absolute numbers of infiltrating cells (Figure 5C), namely the absolute numbers of CD8⁺, but not of CD4⁺ T cells, were consistently and significantly reduced in *W^{sh}/W^{sh}* mice (Figure 5D), whereas reconstitution with WT BMMCs fully restored the CD8⁺ T cell number. This was confirmed upon MOG₃₅₋₅₅ peptide in vitro rechallenge of splenocytes and measured by IFN- γ production (data not shown).

These results demonstrated that BMMCs are not only able to restore the clinical disease phenotype and severity in MC-deficient mice but also specifically reconstitute the impaired CD8⁺ T cell response of MC-deficient mice.

CD8⁺ T Cell-MC Interactions Are Bidirectional

Although we had shown that MCs modulate CD8⁺ T cell responses, it remained unclear whether activated CD8⁺ T cell

can, vice versa, influence MCs activities. Because activated T cells can induce maturation of DCs by enhancing surface expression of MHC and costimulatory molecules (Bernhard et al., 2000), we were specifically interested in the effects of MC-T cell interactions on the expression of MHC class I and costimulatory molecules by MCs. Therefore, unpulsed or OVA₂₅₇₋₂₆₄-pulsed MCs were cocultured with CD8⁺ T cells for 48 hr and MCs were analyzed for surface MHC class I expression by flow cytometry. Indeed, antigen-dependent contact with CD8⁺ T cells significantly increased the cell-surface expression of MHC class I on MCs (Figure 6A).

Furthermore, PCR analysis performed on the cDNA of purified MCs after antigen-dependent coculture with CD8⁺ T cells revealed a substantial upregulation of *4-1BB* (Figure 6B). This shows that CD8⁺ T cells responded to their activation by MCs not only by upregulating the MHC expression on MCs but also by stimulating *4-1BB* expression on MCs.

MCs secrete biologically active osteopontin (OPN) (Nagasaka et al., 2008), which plays an important role in regulating T cell responses during inflammation and infection (Hur et al., 2007). Therefore, we finally examined whether OPN is produced by MCs upon contact with CD8⁺ T cell by measuring the amount of OPN in the supernatants of MC-CD8⁺ T cell cocultures (Figure 6C). The upregulation of OPN by MC-CD8⁺ T cell coculture was confirmed on the RNA level (data not shown). These data suggest that CD8⁺ T cells induce MHC class I and *4-1BB* expression on MCs as well as the secretion of OPN and support

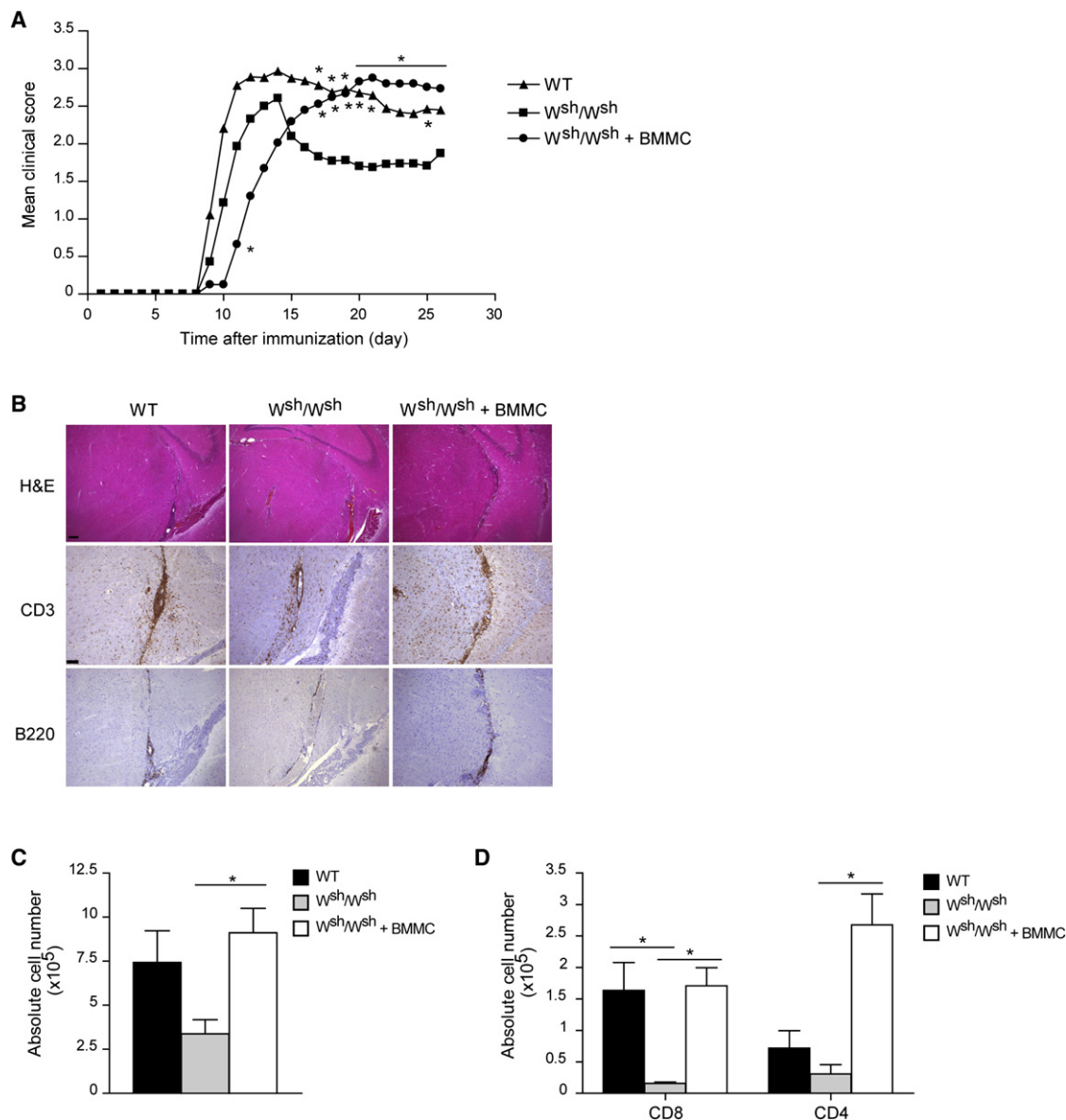


Figure 5. MC Reconstitution in W^{sh}/W^{sh} Mice Restores EAE Severity and Infiltrating CD8⁺ T Cells

(A) Clinical scores of EAE in wild-type (WT) (n = 9), MC-deficient (W^{sh}/W^{sh}) (n = 7), and MC-reconstituted (W^{sh}/W^{sh} + BMBC) (n = 14) mice was assigned daily. Significant differences (p < 0.05) in comparison to W^{sh}/W^{sh} group are indicated by asterisks.

(B) Increased cellular infiltrates in W^{sh}/W^{sh} + BMBC as compared with W^{sh}/W^{sh} mice after EAE induction. Brain sections (thalamus region, day 27) were stained with hematoxylin eosin (H&E) (upper panel). Infiltrating T cells were detected by staining with CD3-specific antibodies (middle panel), and B cells were detected by staining with B220-specific antibodies (lower panel). Naive WT and W^{sh}/W^{sh} controls displayed no infiltrate (Figure S7). Scale bars represent 200 μ m (H&E) and 100 μ m (CD3 and B220).

(C) The absolute number of mononuclear cells in CNS and spinal cord was determined by cell counting and trypan blue exclusion. Mean values $\pm$ SD are shown. (D) Detailed analysis of infiltrating lymphocytes into CNS and spinal cord was performed by flow cytometric analysis of surface marker staining. Mean values $\pm$ SD are shown. *p < 0.01. All panels represent pooled data from two independent experiments.

the concept that MC-CD8⁺ T cell interactions are bidirectional (Figure S8).

DISCUSSION

MCs communicate with CD8⁺ T cells in vivo, namely by inducing CD8⁺ T cell chemotaxis (McLachlan et al., 2003; Orinska et al.,

2005; Ott et al., 2003). MCs also phagocytose bacteria, process bacterial antigens, and induce antigen-specific proliferation of CD8⁺ T cell lines upon presentation of bacterial-expressed proteins in vitro (Malaviya et al., 1996). These observations encouraged us to address whether MCs are capable of inducing antigen-specific CD8⁺ T cell responses in vivo and to characterize the underlying mechanisms of MC-CD8⁺ T cell

Table 1. Cumulative Analysis of EAE Disease Parameters in Mast Cell Reconstitution Experiments

Group	Incidence	Lethality	Mean Day of Onset ^a	Mean High Score
Wild-type	11/11	2/11	9.56 ± 0.18	3.24 ± 0.1 ^b
W ^{sh} /W ^{sh}	9/10	1/10	9.86 ± 0.34	2.7 ± 0.21
W ^{sh} /W ^{sh} + BMMC	15/16	1/16	11.8 ± 0.54	3.26 ± 0.11 ^c

^a Results are expressed as the mean (±SEM).^b $p < 0.03$ as compared with W^{sh}/W^{sh}.^c $p < 0.02$ as compared with W^{sh}/W^{sh}.

interactions, the phenotypical and functional consequences of these interactions, and thus the bidirectionality of this communication.

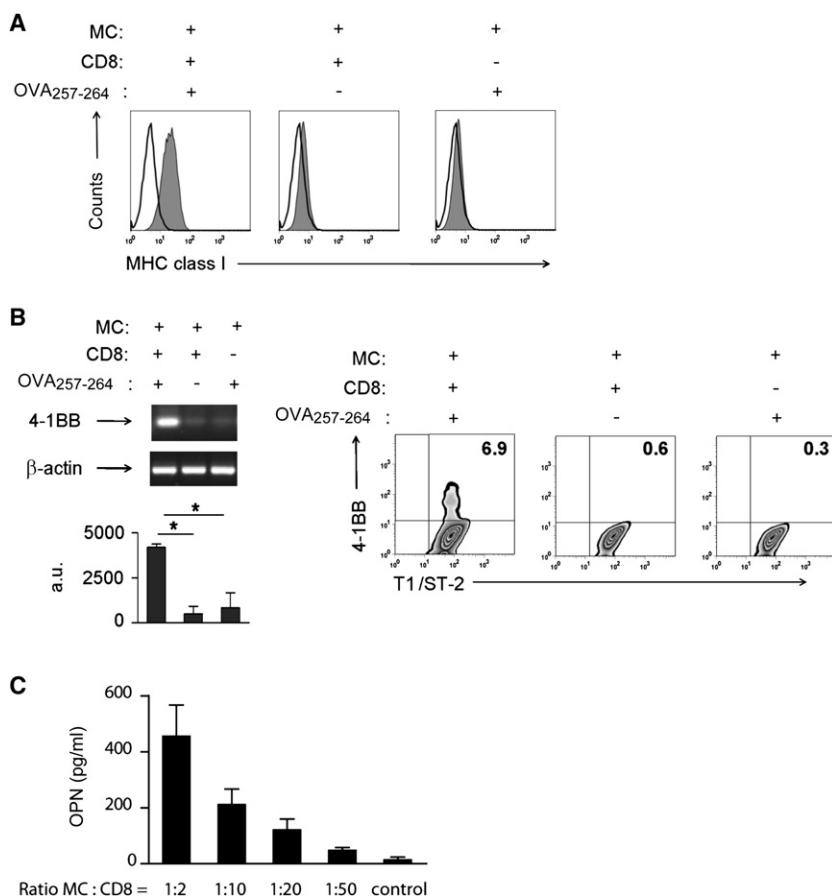
Testing two distinct, well-characterized model antigens (OVA and MOG), we demonstrated here that MCs induce antigen-specific CD8⁺ T cell activation and proliferation in vitro and in vivo. OVA₂₅₇₋₂₆₄ peptide was utilized as a model antigen for studying antigen-presenting functions of different cell types in vitro (Rückert et al., 2003; Winau et al., 2007).

The antigen-specific CD8⁺ T cell response after contact with antigen-pulsed MCs depended on MHC class I-TCR-interactions because MCs with defective MHC class I-presenting functions (i.e., *B2m*^{-/-} MCs) failed to induce substantial CD8⁺ T cell activation, and non-TCR-transgenic CD8⁺ T cells did not respond to the TCR-specific peptide (OVA₂₅₇₋₂₆₄) presented by

MCs. The minimal CD8⁺ T cell response observed after coculture of CD8⁺ T cells with OVA₂₅₇₋₂₆₄-pulsed *B2m*^{-/-} MCs probably reflected residual amounts of peptide remaining in the culture despite extensive washing of the MCs (Schott et al., 2002; Su et al., 1993). MCs also induced CD8⁺ T cell effector functions, such as the production of key T cell cytokines, IFN- γ and IL-2, but none of the Th2 cell-associated cytokines examined (IL-4, IL-5, and IL-10).

Our data confirm and extend the previous proposal that MCs play a dual, complex role in regulating the Th1-Th2 cell cytokine milieu and operate as either predominantly immunosuppressive or immuno-stimulatory cells, depending on the local signaling milieu and/or the strength of costimulatory signals and/or specific signals from the T helper cell environment of MCs (Gregory and Brown, 2006; Stelekati et al., 2007). Our study highlights that, besides their role as inducers of Th2 cell responses through the release of IL-4 (Mekori and Metcalfe, 1999), MCs can also favor Th1 cell responses via interaction with CD8⁺ T cells and induction of IFN- γ and IL-2 production. The latter is potentiated via MC prestimulation by TLR ligands but unaffected by Fc ϵ RI stimulation. This suggests that the stimulation of resident peripheral tissue MCs by pathogen-associated TLR ligands may quickly and effectively promote Th1 cell-type, CD8⁺ T cell-dependent anti-infection defense against such pathogens.

These data conform with the central role of IFN- γ in CD8⁺ T cell cytolytic activity (Andersen et al., 2006). In addition, we show that the intracellular content of Gzmb, a key component of the

**Figure 6. CD8⁺ T Cells Upregulate MHC Class I and 4-1BB Expression in MCs and Induce the Release of OPN In Vitro**

OVA₂₅₇₋₂₆₄-pulsed or unpulsed BMMCs were cocultured with OT-I CD8⁺ T cells (negative control: OVA₂₅₇₋₂₆₄-pulsed BMMCs alone).

(A) After 48 hr, MC surface expression of MHC class I was analyzed by flow cytometry (open histograms: control isotype). Histograms show one representative out of three independent experiments (gated on viable, CD117⁺ MCs).

(B) After 48 hr, MCs were isolated and expression of 4-1BB was tested by PCR. Graph shows mean values of arbitrary units (a.u.) as measured by Optimas Software, $\pm$ SD (three independent experiments). Contour plots show surface expression of 4-1BB on MCs (gated on viable, CD117⁺ MCs). * $p < 0.05$.

(C) The concentration of OPN in the supernatants of MC-CD8⁺ T cell cocultures was measured by ELISA. Graph shows mean values $\pm$ SEM of four independent experiments with at least eight different BMMC cultures.

cytolytic machinery of CD8⁺ T cells, increases in CD8⁺ T cells after antigen-specific activation by MCs and that CD8⁺ T cells degranulate upon antigen presentation by MCs. This concept is in line with the observation that MC-deficient mice fail to initiate peptide-specific cytotoxic responses upon TLR7 activation in vivo (Heib et al., 2007). The MC-dependent CD8⁺ T cell responses revealed here could be important for an optimized host defense, such as protection against intracellular bacteria, because MCs can induce, e.g., activation and proliferation of CD8⁺ T cells upon infection with *L. monocytogenes*. MCs mount an early defense against *L. monocytogenes* by rapid release of TNF- α and by stimulation of neutrophil migration to the site of infection (Gekara and Weiss, 2008). Our data suggest that MCs can, in addition, directly induce effector CD8⁺ T cell activities geared at eliminating intracellular pathogens like *Listeria*.

We showed that MC-CD8⁺ T cell interactions also occur in vivo. Adoptive transfer of antigen-loaded MCs and OT-I transgenic CD8⁺ T cells into *B2m*^{-/-} mice served as a model in which efficient, endogenous MHC-I-restricted antigen presentation is excluded, thus eliminating a contribution of other resident APCs to the antigen-dependent CD8⁺ T cell responses. Furthermore, we provided direct evidence that MCs control disease severity and contribute to CD8⁺-specific priming in EAE and showed that the T cell-proliferation failure is specific to CD8⁺. Our finding that MCs control EAE disease severity in *W^{sh}/W^{sh}* support corresponding findings in *W/W^v* mice (Secor et al., 2000; Tanzola et al., 2003; Gregory et al., 2005), but disagree with the claim that EAE in *W^{sh}/W^{sh}* is indistinguishable from EAE in WT mice (Bennett et al., 2009).

That MCs can induce the proliferation of primary CD8⁺ T cells in an antigen-dependent and antigen-specific manner in vivo supports the concept that MCs function as crucial switchboard cells between innate and adaptive immunity and thereby profoundly modulate adaptive immune responses (Galli et al., 2005; Stelekati et al., 2007; Sayed et al., 2008) and begs the question of whether MCs have similar functions in the human system. Because the majority of MCs resides at peripheral tissue sites (Marshall, 2004; Galli et al., 2008), upon encountering pathogen-related antigens, MCs may internalize the latter to serve as a strategically placed peripheral reservoir for the presentation of pathogen-related antigenic peptides. Indeed, MCs latently infected by human immunodeficiency virus (HIV) constitute a long-lived, inducible peripheral reservoir of viral antigens in humans (Sundstrom et al., 2007). Some MC subpopulations may migrate to regional lymph nodes upon infection or inflammatory stimuli (Dabak et al., 2004; Wang et al., 1998). However, we hypothesize that, in contrast to DCs, which normally execute their antigen-presenting functions in regional lymph nodes, it is primarily resident MCs that play an important, previously unappreciated role in the control of CD8⁺ T cell responses in infection-prone peripheral tissues.

Our study suggests that MCs constitute part of a growing family of nonclassical, functionally important tissue- or microenvironment-specific APCs that complements "professional" APCs (DCs, B cells, and macrophages), such as epithelial cells, fibroblasts, and hepatic stellate cells (Unanue, 2007). Furthermore, our data indicate that MC-CD8⁺ T cell interactions are bidirectional: CD8⁺ T cells also enhance surface MHC class I and

induce 4-1BB expression on MCs as well as the production of OPN after antigen-dependent interaction.

The upregulation of 4-1BB, a costimulatory molecule that promotes proliferation and effector functions of T cells (Hurtado et al., 1995), after antigen-dependent MC contact with CD8⁺ T cells provides mechanistic pointers. MCs can express 4-1BB (Nakae et al., 2006), and 4-1BB-mediated stimulation of MCs enhances cytokine production (Nishimoto et al., 2005). Therefore, increased 4-1BB expression on MCs may enhance the required production and release of cytokines and other soluble factors by MCs. However, it remains to be definitively clarified whether the upregulation of MHC I and 4-1BB on MCs is primarily caused by secreted, T cell-derived factors (e.g., IL-2, IFN- γ) and/or by cell-cell contact-dependent mechanisms. Finally, we showed here that OPN, a key cytokine that supports T cell survival and responses (Hur et al., 2007) and is produced by MCs upon Fc ϵ RI stimulation (Nagasaka et al., 2008), is upregulated and secreted by MCs upon antigen-dependent MC-CD8⁺ T cell interactions. Therefore, the bidirectional crosstalk between MCs and CD8⁺ T cell may not only support CD8⁺ T cell effector functions and cytotoxicity but also regulate CD8⁺ T cell survival.

In summary, we showed that MCs regulate antigen-specific responses of primary CD8⁺ T cells in vitro and in vivo in a bidirectional and cell-cell contact-dependent manner. This MC function is likely to be important in all scenarios in which effector CD8⁺ T cells are critically involved, e.g., infections with viruses and other intracellular pathogens and/or CD8⁺ T cell-dependent autoimmune diseases such as, e.g., multiple sclerosis, alopecia areata, and type I diabetes (Gregory et al., 2005; Gilhar et al., 2007; Sayed et al., 2008). It now deserves systematic scrutiny whether, in these settings, the stimulation or inhibition of MC-CD8⁺ T cell interactions offers a promising new target for more effective therapeutic management.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 WT mice were purchased from Charles River (Sulzfeld). TCR-transgenic OT-I mice (Hogquist et al., 1994) and *I12*^{-/-} mice (Schorle et al., 1991) were bred in the Animal Care Facility of the Research Center Borstel. *B2m*^{-/-} mice (Koller and Smithies, 1989) were kindly provided by F. Winau (MPI for Infection Biology, Berlin). *Ifng*^{-/-} (B6.129S7-*Ifng*^{tm1Ts/J}), *Ccl3*^{-/-} (B6.129P2-*Ccl3*^{tm1UNC/J}), and *Kit*^{W-sh} (B6.Cg-*Kit*^{W-sh/HNhrJaeBsmJ}) (*W^{sh}/W^{sh}*) mice were from Jackson Laboratory. All in vivo animal experiments were conducted with permission and in accordance with current federal, state, and institutional guidelines.

Generation of BMMCs and BMDCs

Murine bone marrow cells were cultivated in IMDM complete medium with 10% FCS (Biochrom), 5 ng/ml IL-3 (rIL-3), and 10 ng/ml stem cell factor (rSCF) (both from R&D) (Huff and Lantz, 1997). After 5 weeks of culture, BMMCs (CD117⁺ (c-Kit), Fc ϵ RI⁺, T1/ST2⁺, CD11c⁻, CD45R/B220⁻, F4/80⁻, and Gr-1⁻) represented >98% of the total cells. BMDCs were generated by cultivation of bone marrow cells in the presence of GM-CSF as described (Lutz et al., 1999).

Mast Cell Reconstitution of *W^{sh}/W^{sh}* Mice

Six-week-old *W^{sh}/W^{sh}* mice were reconstituted with 4×10^6 wild-type BMMCs injected intravenously. Four weeks after reconstitution, immunization with MOG and induction of EAE were performed. MC reconstitution was analyzed by staining of formalin-fixed spleen sections with Napthol AS-D chloroacetate (Sigma).

Flow Cytometry

Cells were resuspended in FACS-buffer (2% newborn calf serum, 0.1% Na₂S₂O₈, 2 mM EDTA in PBS), stained with mAb against CD3 ϵ (145-2C11), CD4 (RM4-5), CD8 α (53-6.7), CD45R/B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11c (HL3), CD11b (M1/70), CD49b/Pan-NK (DX5), CD69 (H1.2F3), CD25 (PL61), CD44 (IM7), CD117/c-kit (2B8), Fc ϵ RI (MAR-1), H2-K^b (AF6-88.5) (all from BD Pharmingen), and CD137/4-1BB (17B5), MHC class I OVA peptide (25-D1.16) (eBioscience), T1/ST2 (DJ8) (Morwell Diagnostics), and F4/80 (MCA497FB) (Serotec) for 20 min at 4° C, and subsequently washed with 1 μ g/ml propidium iodide (Sigma-Aldrich) in FACS buffer. For measuring degranulation of CD8⁺ T cells, rat anti-mouse LAMP-1 (1D4B) mAb (DSHB, University of Iowa) and F(ab')₂ anti-rat IgG secondary antibody (Serotec) were used. For staining of CD137 and H2-K^b on MCs, cells were incubated with anti-CD16/32 (BD Pharmingen) prior to the addition of the mAb for surface staining. Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Intracellular flow cytometry staining was performed in accordance with a standard protocol from eBioscience. After 6 hr of cell culture with 10 μ g/ml brefeldin A (Sigma), cells were washed and surface FACS staining was performed. After fixation and permeabilization, intracellular staining was performed by incubation with mAb against IL-2 (JE96-5H4), IFN- γ (XMG1.2), and TNF- α (MP6-XT22) (BD Pharmingen), MIP-1 α (R&D Systems), or Gzmb (16G6) (eBioscience). All plots were gated on propidium iodide-negative CD8⁺ T cells.

Purification and Coculture of CD8⁺ T Cells, MCs, and Macrophages

CD8⁺ T cells were isolated from lymph nodes of OT-I TCR-transgenic mice by negative selection with magnetic-associated cell sorting (MACS). Cells were stained with FITC mAb against CD4, CD45R/B220, CD49b/Pan-NK, CD11c, CD11b, and F4/80, washed, incubated with magnetic anti-FITC-labeled microbeads (Miltenyi Biotec), and sorted with autoMACS (Miltenyi Biotec). The sorting yielded >98% CD8⁺ T cells.

For purification of MCs after the in vitro coculture with CD8⁺ T cells, magnetic-associated positive selection was used. Cells were incubated with biotin-labeled c-kit specific mAb, washed, incubated with streptavidin-micobeads, and selected with autoMACS. The purity of the MC fraction was >98%, as shown by CD117, T1/ST2, and Fc ϵ RI expression, determined by FACS.

Peritoneal cells from WT mice were isolated by peritoneal lavage with 10 ml of ice-cold 0.9% NaCl solution. Peritoneal mast cells (PMCs) were selected in a FACS sorter (FACS Aria, BD Biosciences) in accordance with their expression of CD117 and T1/ST2. Purity of PMCs was >98%, according to toluidine blue staining.

Primary macrophages were selected from cells of peritoneal lavage by adherence to tissue type cell culture plates for 2 hr. Nonadherent cells were washed away and macrophages stimulated in vitro with 20 μ g/ml OVA before the CD8⁺ T cells at a ratio MF:CD8 1:10 were added.

MCs were incubated with 4 μ g/ml OVA₂₅₇₋₂₆₄ (NeoMPS) for 3 hr or with 100 μ g/ml OVA (OVA grade VI, Sigma) for 8 hr at 37°C and washed four times. OVA₂₅₇₋₂₆₄ was tested for endotoxin activity by Coamatic Chromo-LAL kit (Hemochrom Diagnostica, Essen, Germany) with a resolution limit of 0.005 EU/ml. These were in a range of 0.6–0.7 EU/ml corresponding to 40–47 pg/ml. Furthermore, the TLR2 agonistic activity of the OVA₂₅₇₋₂₆₄ peptide was measured by induction of IL-8 production in cells transiently transfected with TLR2 as described (Heine et al., 2007), and this was lower than 0.1 nM TLR2 agonist Pam₃CSK₄. CD8⁺ T cells were plated at a density of 2 $\times$ 10⁶ cells/ml and BMMCs were added at different ratios. In some experimental conditions, the BMMCs were pretreated overnight with 2 μ g/ml of IgE (SP7, Sigma) before OVA₂₅₇₋₂₆₄ loading and then the IgE-prensensitized BMMCs were cultured in the presence of Ag 100 ng/ml (DNP-HSA, Sigma).

For proliferation assays, CD8⁺ T cells were labeled with 3 μ M CFSE (Vybrant CFDA SE, Cell Tracker Kit, Molecular Probes) for 6 min at room temperature. The reaction was stopped by addition of FCS, cells were washed, and proliferation was measured by FACS 72 hr later.

Concentrations of cytokines were determined by specific ELISAs provided by R&D Systems.

Infection of BMMCs with *Listeria monocytogenes*

WT *Listeria monocytogenes* (strain EGD) or recombinant strain *Listeria monocytogenes*-OVA (Foulds et al., 2002) were cultured overnight, and bacterial

density was determined by absorption at 600 nm. BMMCs were washed and resuspended in IMDM complete medium without antibiotics, at a concentration of 1 $\times$ 10⁶ cells/ml. *Listeria monocytogenes* or *Listeria monocytogenes* OVA were added at a MOI of 1:1–100:1 for 1 hr at 37°C. Next, 50 μ g/ml gentamycin (GIBCO-Invitrogen) was added to the BMMCs for 30 min, and BMMCs were washed three times and cocultured with the CD8⁺ T cells in IMDM medium containing 10 μ g/ml gentamycin.

Adoptive Transfer Experiments

A total of 5 $\times$ 10⁶ BMMCs were incubated with 4 μ g/ml OVA₂₅₇₋₂₆₄ for 3 hr at 37°C and after four washings were injected intraperitoneally in WT or B2m^{−/−} mice. One day later, purified OT-I CD8⁺ T cells were labeled with CFSE and injected in the peritoneum of recipient mice (8 $\times$ 10⁶ cells/mouse). Four days later, proliferation of transgenic CD8⁺ T cells was analyzed by CFSE dilution.

EAE Induction, Clinical Score, and Histological Analysis

EAE was induced in mice by injection subcutaneously in both flanks with a total of 200 μ g of MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) (NeoMPS) dissolved in PBS emulsified in an equal volume of CFA (Difco) supplemented with 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco). Mice were injected twice intravenously with 300 ng of pertussis toxin (Calbiochem) administered on the day of immunization and 48 hr later. Mice were scored daily as follows: 0, no detectable signs of EAE; 0.5, distal limb tail; 1.0, complete limb tail; 1.5, limb tail and hind-limb weakness; 2, unilateral partial hind-limb paralysis; 2.5, bilateral partial hind-limb paralysis; 3, complete bilateral hind-limb paralysis; 3.5, complete hind-limb paralysis and unilateral forelimb paralysis; 4, total paralysis of forelimbs and hind limbs; and 5, death. Immunohistochemical stainings were carried out as described (Kellner et al., 2009). In brief, paraffin-embedded sections from brain were stained with hematoxylin eosin (H&E), giemsa, or immunostaining to T cells (CD3, Dako) and B cells (B220, E-Bioscience), with horseradish peroxidase-labeled secondary antibodies (Nichirei) and diaminobenzidine as chromogen. For the preparation of CNS lymphocytes, mice were perfused through the left cardiac ventricle with PBS. Afterward, brain and spinal cord were removed and incubated 30 min in a digestion solution (1 mg/ml collagenase and 0.1 mg/ml DNase from Sigma-Aldrich). Subsequently, the digested tissue was transferred on a cell strainer and the RBCs were lysed. CNS mononuclear cells were isolated by a Percoll gradient (1.095 g/ml–1.030 g/ml) from GE-Healthcare. The isolated cells were washed and resuspended in staining buffer for a direct cell-surface staining.

Ag Recall Proliferation

Seven days after immunization, mice were sacrificed and spleens were removed. For the T cell stimulation in vitro, BMDcs and BMMCs were preloaded with 5 μ M of MOG₃₅₋₅₅ peptide for 2 hr at 37°C and then CFSE-labeled CD8⁺ T cells or splenocytes were added at a ratio 1:10. After 3 days, the cells were washed and cultured for 2 days in 50 ng/ml of recombinant IL-2. The proliferation was measured by FACS.

Real-Time PCR

RNA was extracted from cells with TRIZOL reagent (GIBCO-Invitrogen). Semi-quantitative RT-PCR analysis was done with primers as follows: mouse 4-1BB sense: 5'-AGT GTC CTG TGC ATG TGA-3' antisense: 5'-AGT TAT CAC AGG AGT TCT GC-3', mouse OPN sense: 5'-GCA CCC AGA TCC TAT AGC-3', antisense: 5'-TCC GTT GTT GTC CTG ATC-3' and mouse β -actin sense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3', antisense: 5'-GTG GGG CGC CCC AGG CAC CA-3'. The number of cycles in the RT-PCR was 30.

Statistical Analysis

Data are represented as mean $\pm$ standard deviation (SD) or standard error of mean (SEM) as indicated. Statistical significance was tested by Student's t test for unpaired samples. A p value of < 0.05 was considered as statistically significant.

SUPPLEMENTAL DATA

Supplemental Data include eight figures and can be found with this article online at [http://www.cell.com/immunity/supplemental/S1074-7613\(09\)00409-9](http://www.cell.com/immunity/supplemental/S1074-7613(09)00409-9).

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